Cornulin, a New Member of the ''Fused Gene'' Family, Is Expressed During Epidermal Differentiation

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The protein encoded by the *C1orf10* gene was described to be esophageal-specific and a marker for cancer development. This protein, however, has the previously unreported structural features of the ''fused gene'' family combining sequences and structural similarities of both the S100 proteins and precursor proteins of the cornified cell envelope as in profilaggrin, trichohyalin, and repetin. Since all members of this family are expressed in keratinocytes, we suspected a role in epidermal differentiation and named the protein cornulin. Here, we report that human cornulin mRNA is expressed primarily in the upper layers of differentiated squamous tissues including the epidermis. Using polyclonal peptide antibodies, we show that cornulin is expressed in the granular and lower cornified cell layers of scalp epidermis and foreskin, as well as in calcium-induced differentiated cultured keratinocytes. Ca^{2+}-overlay assay indicated that EF-hand domains of cornulin are functional and bind calcium. In HeLa cells, cornulin, co-transfected with transglutaminase 1, was diffusely distributed throughout the cytoplasm in contrast to small proline-rich 4, which localized to the cell periphery. We conclude that cornulin is a new member of the ''fused gene'' family, does not appear to be a precursor of the cornified cell envelope by itself, and is a marker of late epidermal differentiation.

Key words: epidermis/keratinocytes/differentiation complex/1q21/fused gene
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The skin is the first line of defense *versus* chemical, physical, and microbial insults. Because of these perpetual attacks, the epidermis is continuously regenerating through a process called epidermal differentiation. The terminal differentiation of a keratinocyte in the epidermis involves the cessation of proliferation and subsequent migration from the basal layer to the suprabasal layers with progressive cornification. The cornified outermost cell layer provides a barrier that resists various insults from the external environment and prevents water loss (Kalinin *et al*, 2002). Epidermal differentiation is a complex process that requires a regulated and sequential expression of a variety of genes. Each epidermal layer is characterized by the expression of specific markers such as keratins K5/K14 in the basal cells (Moll *et al*, 1982), K1/K10 in the spinous cells (Fuchs and Green, 1980), and K2e in the granular cells. Several genes involved in epidermal differentiation are found within a 2-Mb region at chromosome band 1q21 called the epidermal differentiation complex (EDC) (Mischke *et al*, 1996; Lioumi *et al*, 1998; South *et al*, 1999). During the final stages of differentiation, these specific proteins are cross-linked together by the action of transglutaminases (TG) to form the cornified envelope.

The EDC contains three clustered families of genes encoding the following: (a) precursor proteins of the cornified cell envelope (involucrin, loricrin, and the small proline-rich (SPRR) proteins), characterized by short tandem peptide repeats in the central region (Mehrel *et al*, 1990; Haftek *et al*, 1991; Marvin *et al*, 1992; Steinert and Marekov, 1995); (b) calcium-binding proteins (S100 proteins), containing EF-hand domains (Haftek *et al*, 1991; Presland *et al*, 1992; Lee *et al*, 1993; Markova *et al*, 1993; Steinert and Marekov, 1995; Krieg *et al*, 1997); and (c) a family of proteins (profilaggrin, trichohyalin, hornerin, repetin), which combine EF-hand domains at the N-terminus followed by multiple tandem peptide repeats and thus have been described as "fused genes" (Presland *et al*, 1992; Markova *et al*, 1993; Krieg *et al*, 1997).

Recently, a novel gene, *c1orf10*, located on 1q21 was described as an esophageal-specific and cancer-associated gene (Xu *et al*, 2000; Luo *et al*, 2003). *C1orf10* product is a protein of 495 amino acids with a calcium-binding motif of about 90 residues at its N-terminus and a conserved consecutive repeat sequence of 60 amino acids (Xu *et al*, 2000). Its structural characteristics are similar to those of "fused gene" family members of the EDC, but were not recognized. Here, we have characterized the expression of *c1orf10* gene product, which we named cornulin, in human tissues, particularly the foreskin. We demonstrate that the "fused gene" family member cornulin is a Ca^{2+}-binding protein present in the upper layer of squamous epithelia. Therefore, cornulin might have an important role in epidermal differentiation.

Abbreviations: cDNA, complementary DNA; DIG, digoxigenin; EDC, epidermal differentiation complex; PBS, phosphate-buffered saline; TG, transglutaminase

Results

Analysis of 5′ upstream DNA sequence and gene structure of human cornulin It has been shown that *c1orf10*,

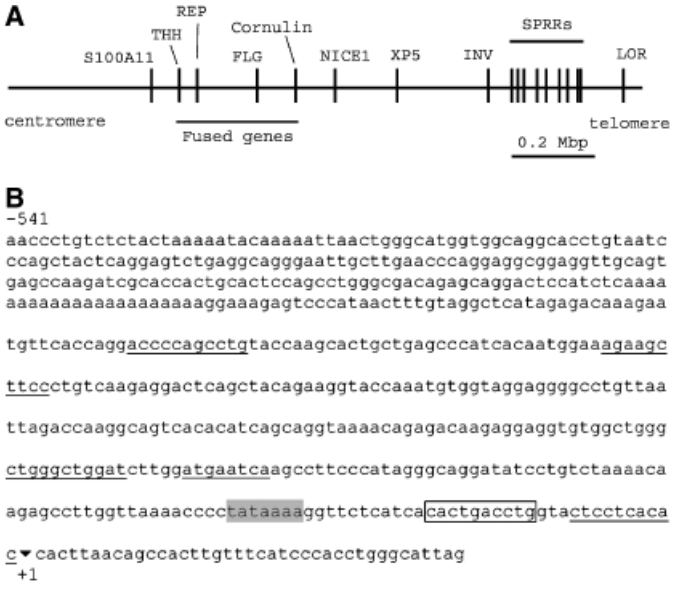

Figure 1
The "fused gene" family is clustered between S100A11 and NICE1 genes. (*A*) THH, trichohyalin; REP, repetin; FLG, filaggrin; NICE-1, newly identified cornified envelope protein 1; INV, involucrin; SPRR, small proline-rich proteins; and LOR, loricrin. (*B*) A portion of 5′ end upstream exon 1 of cornulin is shown. The probable TATA box element is *shaded* and the transcription initiation site is indicated by an *arrowhead*. AP1 site is *double-underlined*, and several SP1 sites are *underlined*. A predicted RA-response element is *boxed*.

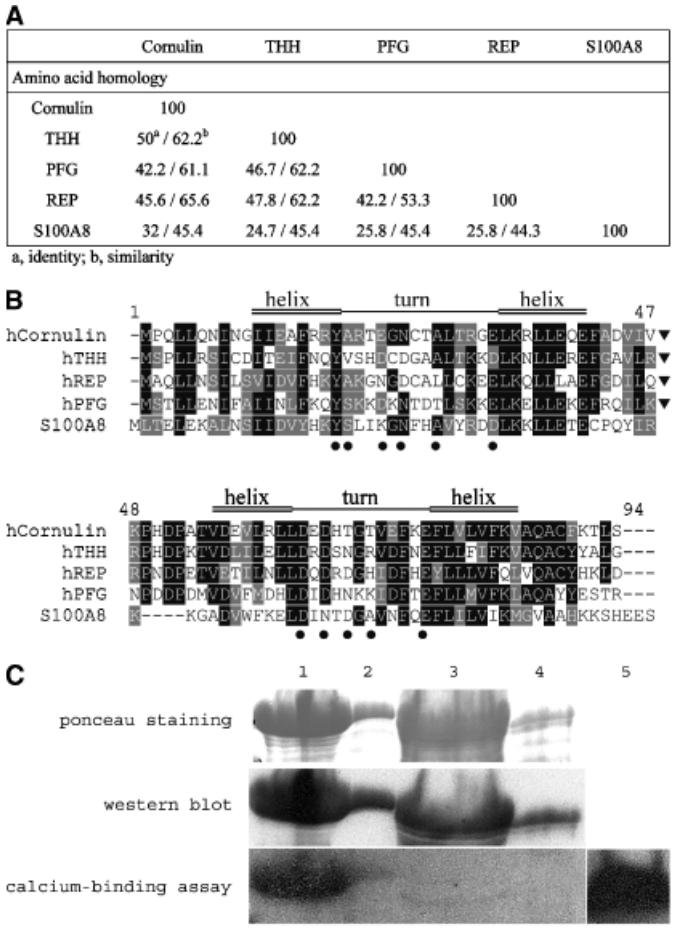

Figure 2
Human cornulin is able to bind Ca^{2+}. (*A*) Percentage of homology among the amino-terminal 90 residues of "fused gene" family members. (*B*) Multiple alignment of the amino-terminal 90 amino acids of human cornulin, trichohyalin (hTHH), repetin (hREP), profilaggrin (hPFG), and S100A8. Identical residues are *black-shaded*, and similar residues are *gray-shaded*. The helix-turn-helix sequences that define each EF-hand motif are shown. The position of the intron between the EF hands is indicated by an *arrowhead*. Residues essential for calcium binding to S100A8 (Ishikawa *et al*, 2000) are shown by *dots* below the sequence. (*C*) $^{45}Ca^{2+}$ overlay assay. Lanes 1 and 2, soluble and particulate fraction from bacteria transformed with pET28C, respectively. Lanes 3 and 4, soluble and particulate fraction from bacteria transformed with pET28CΔEF, respectively. Lane 5, bovine brain calmodulin (10 μg). Proteins were separated by SDS-PAGE, transferred onto nitrocellulose membrane, and incubated with $^{45}Ca^{2+}$.

encoding cornulin, was located on chromosome 1 in region q21 (Xu *et al*, 2000). Using the human genome sequence, we have established that it is located within the epidermal differentiation complex, very close to the other members of the "fused gene" family (profilaggrin, trichohyalin, and repetin). This family is organized in a cluster of ~ 300 kb in the EDC, between S100A11 and NICE1 (Marenholz *et al*, 2001) genes (Fig 1*A*).

In silico analysis of the region 5′ upstream the transcription start site allowed us to identify a retinoic acid (RA) response element (RAR) at position −23, a TATA-box binding protein (TBP) site at position –41 as well as many SP1 sites, located in intron 1, non-coding exon 1 and all around the TBP site. Moreover, a perfect consensus AP1 regulatory motif, described in Jang *et al* (1996), was also detected at position −105 (Fig 1*B*).

C1orf10 gene structure is similar to that of the "fused gene" members, i.e., a short first non-coding exon; a long intron 1; a second exon containing the initiation codon and encoding the first EF-hand motif; intron 2; a third exon containing the second EF-hand motif and the additional coding sequence. The main difference between cornulin and other "fused gene" members reside in the number and length of repetitive units. Cornulin has only two repeats of 60 amino acids while other fused gene members have variable motifs repeated several times.

Human cornulin binds Ca^{2+} Multiple sequence alignment analysis shows that the N-terminal region (90 amino acid) of cornulin, containing two EF-hand domains, is closely related to the same region of other "fused proteins" such as repetin, profilaggrin, and trichohyalin and, to a lesser extent, to sequences of S100 protein family (Xu *et al*, 2000) (Fig 2*A*). All these proteins bind calcium. Sequence alignment with the most similar S100 member S100A8, for which a three-dimensional structure is available (Ishikawa *et al*, 2000), indicates that the residues implicated in the binding of calcium are rather well conserved in cornulin (Fig 2*B*). Therefore, we tested whether cornulin could bind calcium as well. Thus, cornulin and cornulinΔEF were expressed in bacteria using the pET28a vector. Soluble and particulate fractions from isopropyl β-D-thiogalactoside (IPTG)-induced cultures of p28C and p28C ΔEF were electrophoresed and blotted to a membrane. The presence of calcium-binding proteins was tested by an $^{45}Ca^{2+}$ overlay assay, as previously described (Maruyama *et al*, 1984). Autoradiography of the membrane (Fig 2*C*) revealed the presence of a radioactive band whose position coincides with the band of

A

	hCornulin	mCornulin	rCornulin	cCornulin
Amino acid homology				
hCornulin	100			
mCornulin	51.1[a] / 57.6[b]	100		
rCornulin	51.3 / 56.9	81.9 / 84.6	100	
cCornulin	74.5 / 77.1	44.8 / 51.4	43.8 / 49.4	100

a, identity; b, similarity

B

Figure 3
Cornulin protein sequence is well conserved in different mammalian species. (*A*) Percentage of homology among cornulin from the following species: h, human; m, mouse (accession number XM_355410); r, rat (XP_227367); and c, chimp. (*B*) Alignment, using AlignX program, of the highly conserved amino-terminal sequence of cornulin from the different species.

cornulin visualized by both Ponceau red staining and western blot analysis (Fig 2*C*, *lane 1*). In contrast, no radioactive signal is visible at the position of cornulin lacking the EF-hand domains (Fig 2*C*, *lane 3*). Bovine brain calmodulin (Calbiochem Dietikon, Switzerland) was used as positive control (Fig 2*C*, *lane 5*). Thus, this result demonstrates that cornulin binds Ca^{2+}.

Cornulin homologues in other species Cornulin Blast searches in the Ensembl databases allowed us to identify a murine homologue on mouse chromosome 3. We cloned this homologue, whose coding sequence was identical to that contained in GenBank (accession number XM_355410). Protein sequence comparisons determined that human cornulin presents 57.8% similarity to mouse cornulin. Furthermore, a putative rat homologue on rat chromosome 2 (accession number XP_227367) and a putative chimp homologue were also found (Fig 3*A*). Interestingly, multiple sequence alignment analysis showed that the amino-terminal region containing the EF-hand domains is the most conserved among all four species with 82.6% identity (Fig 3*B*). This result underlines the importance of the calcium-binding domain for the function of cornulin.

Tissue expression of human cornulin RT-PCR experiments showed that the cornulin transcript was expressed in fetal bladder, scalp skin, foreskin, and in cultured primary keratinocytes (Fig 4*A*). Traces of cornulin mRNA were found in fetal brain, adult lung, kidney, uterus, skeletal muscle, and heart. Cornulin mRNA was more abundant in scalp skin and foreskin (Fig 4*A*) than in other tissues.

Semi-quantitative RT-PCR experiments using 25, 30, and 35 PCR cycles showed that cornulin mRNA is more abundant in differentiated cultured keratinocytes, 7 d after shifting from 0.1 to 1.2 mM Ca^{2+} (Fig 4*B*). This was confirmed both by northern blot analysis, which showed a strong increase of expression, after the Ca^{2+} shift, of the 1.9 kb cornulin mRNA (Fig 4*C*), and by ISH analysis, which indicated that cornulin mRNA is expressed in the upper layers of the epidermis (Fig 4*D*).

Complementary DNA constructs and localization of cornulin in HeLa cells To investigate the cellular localization of cornulin, we constructed three expression vectors, giving rise to N-terminus GFP fusion proteins as follows: (a) full-length cornulin (pEGFP-cornulin), (b) deletion of 89 first N-terminus amino acids containing the EF-hands (pEGFP-cornulinΔEF), and finally (c) N-terminus containing EF-hands only (pEGFP-EFh). Furthermore, two other full-length constructs of cornulin were generated with either a HA or a V5 tag at N- or C-terminal position, respectively (Fig 5*A*).

Confocal microscopy of HeLa cells transfected with pEGFP-Cornulin revealed a cytoplasmic distribution of cornulin with a denser staining around the nucleus (Fig 5*B*). In contrast, pEGFP-CornulinΔEF exhibited mostly a granular pattern in the whole cell. pEGFP-EFh localized both in the cytoplasm and in the nucleus, as observed for GFP control.

Concerning the possibility that cornulin was a substrate of TG, no colocalization of TG 1 and cornulin was observed after co-transfection of expression vectors in HeLa cells. This indicates that cornulin is not processed by TG 1 in contrast to SPRR4, shown to be cross-linked by TG 1 and thus localized to the cell periphery (Cabral *et al*, 2001) (Fig 5*B*).

Characterization of anti-cornulin antibody A polyclonal antibody, SZ1229, was produced against a sequence located in the repeats region (EATNDQNRGTETHGQG) and was affinity purified. The predicted molecular weight of cornulin is 53 kDa. Western blot analysis performed on total extracts from 293T cells, transfected with different tagged-fusion vectors (GFP, HA, and V5), revealed that both anti-tag and anti-cornulin (SZ1229) antibodies recognized the same proteins (Fig 6*A*). Anti-GFP antibody detected a protein that migrated at about 100 kDa, while anti-HA and anti-V5 antibodies detected a protein at about 70 kDa. As the calculated weight is about 83.5 kDa for the GFP-tagged cornulin, and about 56.5 kDa for HA and V5-cornulin, we can hypothesize that the apparent molecular weight is due either to an aberrant migration or to the fact that our protein is subject to post-translational modifications, the nature of which is not yet known. However, unlike profilaggrin, our experiments did not give any evidence of proteolytic processing *in vivo* (Fig 6*B*, *left panel*).

Time-course expression analyses in human keratinocytes revealed a unique 70 kDa immunogenic protein in the d7 cytosolic extract (Fig 6*B*, *right panel*). Preincubation of our antibody with a 100 × excess of peptide epitope led to the disappearance of the 70 kDa band, confirming the peptide specificity of SZ1229 (Fig 6*C*). At d7, keratinocytes were differentiated, as indicated by the expression of K10. Thus, we can say that cornulin, like the other "fused" proteins, is a marker of late differentiation.

Expression of cornulin antigen in human epidermis Expression in different epithelial tissues was investigated by

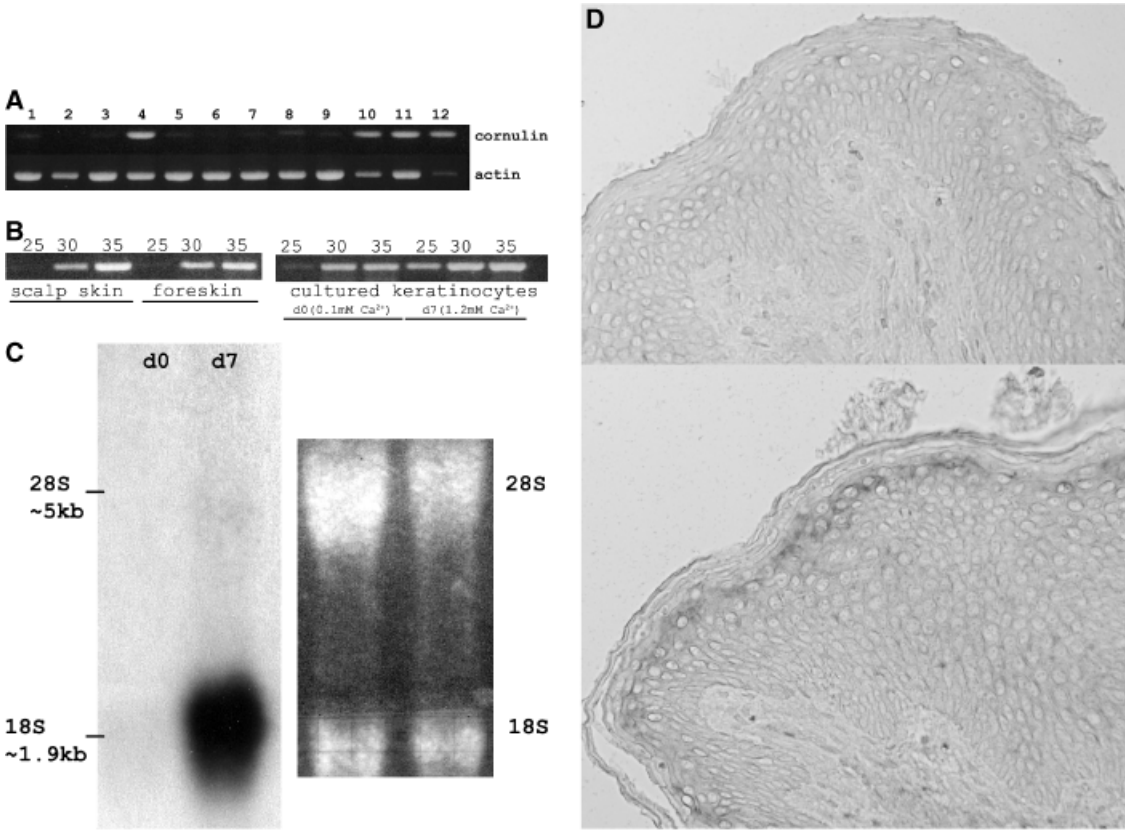

Figure 4
Cornulin mRNA is primarily expressed in upper layers of squamous tissues. (*A*) RT-PCR experiments were performed using total RNA from various human tissues with 3′UTR specific primers of cornulin (upper panel) and actin primers (bottom panel). Lanes 1, fetal brain; 2, adult liver; 3, adult lung; 4, fetal bladder; 5, adult kidney; 6, adult colon; 7, adult uterus; 8, adult skeletal muscle; 9, adult heart; 10, scalp skin; 11, cultured keratinocytes; 12, foreskin. (*B*) cDNA samples were submitted to 25, 30, and 35 PCR cycles. Cornulin transcript is more abundant in the cultured keratinocytes, especially in Ca^{2+}-induced differentiating cells. (*C*) Northern-blot analysis of cornulin transcript in proliferating (d0) or differentiating (d7) cells after Ca^{2+} shift from 0.1 to 1.2 mM. Total RNA (12 μg) was isolated, electrophoresed, blotted, and hybridized with a [^{32}P]dATP-labeled cornulin probe. Bands of 28S and 18S rRNA were used as controls for the loading of total RNA. (*D*) *In situ* detection of cornulin mRNA in human foreskin. Sections of human foreskin were hybridized with digoxigenin (DIG)-labeled cornulin sense (*top*) and antisense (*bottom*) RNA probes. A positive signal is clearly visible, with the antisense probe, in the granular layer of the epidermis.

immunofluorescence analysis using the affinity-purified antibody SZ1229 (Fig 7). Experiments performed on foreskin sections (Fig 7*A–C*) exhibited a peripheral immunolabeling of the granular and lower cornified cells (Fig 7*A*, *B*). Cornulin expression was also found in scalp skin (Fig 7*D–F*) in the granular layer and the inner root sheath of the hair follicle. No staining was visible in control sections (Fig 7*C* and *G*). Finally, prominent staining was observed on esophagus sections at the periphery of the cells of the granular and the upper spinous layers (Fig 7*H*).

Discussion

In this paper, we have analyzed the gene *c1orf10* and its protein product, which we called cornulin, previously described as being esophagus-specific (Xu *et al*, 2000). We present several lines of evidence that cornulin belongs to the "fused gene" family of proteins and plays a role in squamous differentiation. *C1orf10* is located within the EDC and possesses the genomic and structural characteristics of a "fused gene" family member: (1) it is located on chromosome 1q21, (2) it contains three exons (exon 1 is non-translated), and (3) it encodes a protein with the fused protein structure, i.e., an amino-terminal Ca^{2+}-binding domain and repeats in the carboxy-terminal region.

Structurally, cornulin contains two EF-hand Ca^{2+} binding domains in its amino-terminal region highly comparable with those of profilaggrin (Presland *et al*, 1992), trichohyalin (Lee *et al*, 1993), repetin[1], and mouse hornerin (Makino *et al*, 2001). Cornulin also bears two repeats of 60 amino acids in the C-terminal part (Xu *et al*, 2000), which are rich in glutamine ($\sim$ 24%) and threonine ($\sim$ 21%). They are mainly made of polar ($\sim$ 57%) and charged ($\sim$ 25%) residues. The function of these two repeats remains unknown. Concerning its amino-acid composition, cornulin is more similar to repetin, another member of the "fused gene" family, known to be implicated in epidermal differentiation (Krieg *et al*, 1997).

Despite strong expression in the esophagus, cornulin is not specific for this tissue and both transcript and protein are found in scalp skin and foreskin, as well as in cultured keratinocytes. Furthermore, as for profilaggrin and other "fused gene" proteins, Ca^{2+} could play a role in the

[1] Huber *et al*, manuscript submitted.

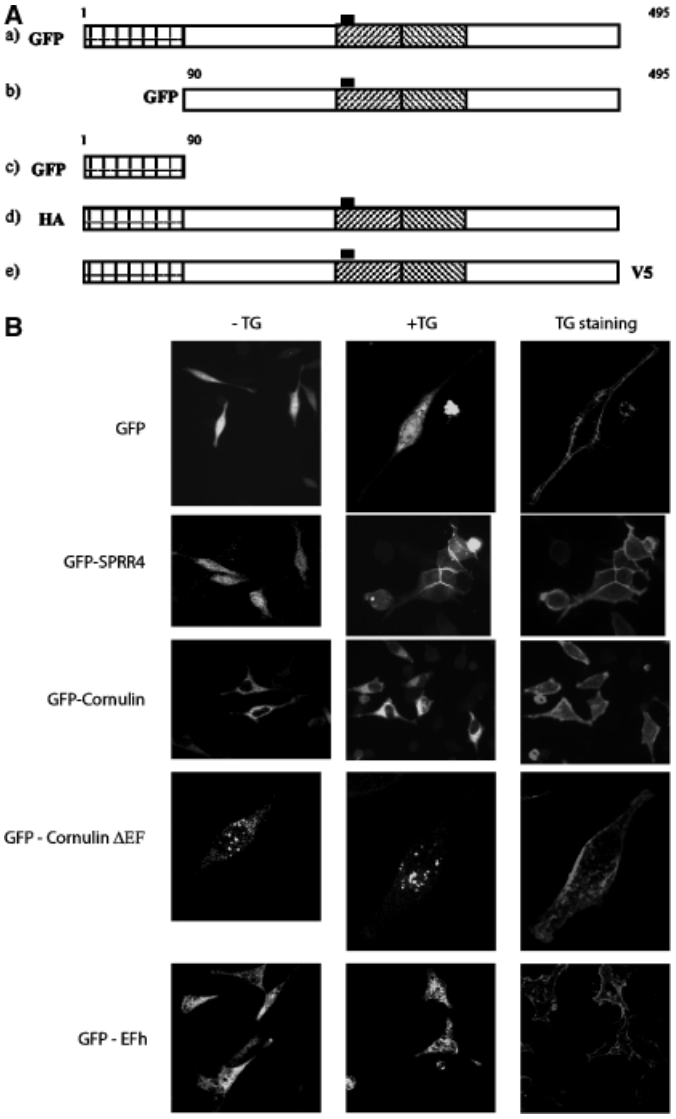

Figure 5
***(A)* Constructs of expression vectors**. (*a*) GFP-cornulin full length, (*b*) GFP-cornulinΔEF, (*c*) GFP-EF-hand domain of cornulin, (*d*) HA-cornulin and (*e*) V5-Cornulin. Amino-terminal region containing EF-hand domains (*squared box*) and repeats (*diagonal hatched boxes*) are indicated as well as epitope site (*horizontal black bar*). (*B*) Cornulin is cytoplasmic and not a substrate for TG 1. HeLa cells were co-transfected with the indicated constructs and fixed. Cellular distribution was examined by GFP autofluorescence (*left panel*) and by staining with a mouse anti-TG 1 antibody (*right panel*). Cornulin has a cytoplasmic distribution and is not a substrate of TG 1, unlike control SPRR4 that colocalizes with this enzyme in the cell periphery. Control GFP exhibits a diffuse epifluorescence throughout the cytoplasm and nucleus.

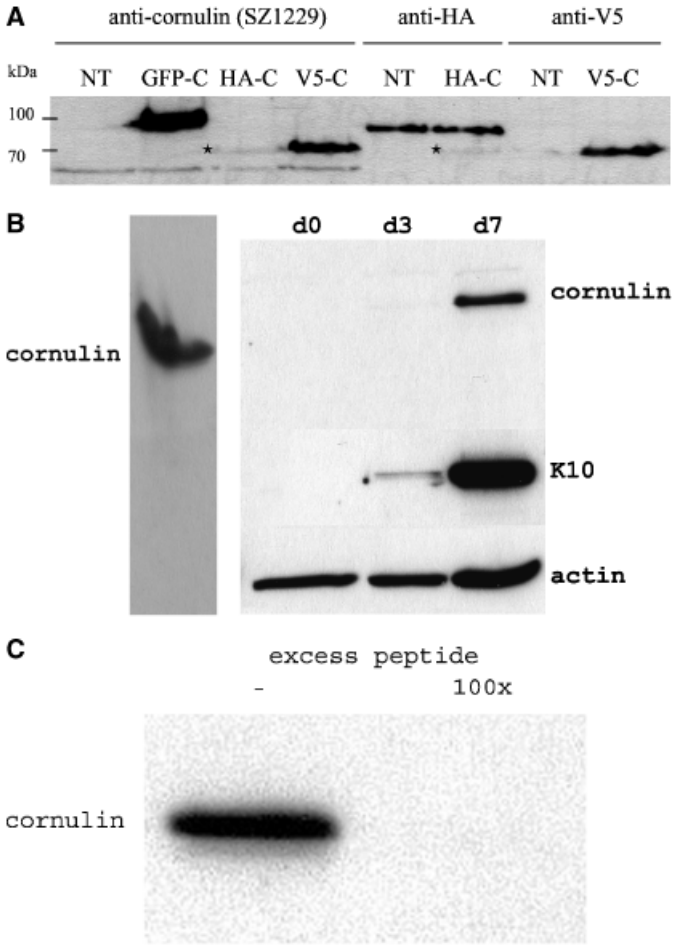

Figure 6
Cornulin expression *in vitro* is induced by calcium. (*A*) Characterization of SZ1229 antibody with total extracts of 293T cells transfected with our different tagged-cornulin vectors. Each fusion protein was detected with both anti-tag and anti-cornulin (SZ1229) antibodies. Western blot analysis shows the specificity of SZ1229. NT, non-transfected cells; black stars indicate specific band for HA-C. (*B*) Western blot was performed with total foreskin extract (*left panel*) or with cytosolic extracts (10 μg) of foreskin cultured keratinocytes before (d0) and after (d3 and d7) a Ca^{2+} shift from 0.1 to 1.2 mM (*right panel*). Blot was probed with anti-cornulin antibody (SZ1229). The antibody recognizes cornulin at $\sim$ 70 kDa. *C*) Antigen competition performed on d7 extracts (10 μg). SZ1229 antibody was incubated for 1 h with a 100 $\times$ excess of antigenic peptide prior to cornulin detection by western blotting. Antibody detected cornulin and the band disappeared after the pre-treatment of the antiserum with the antigenic peptide.

regulation of its expression because cornulin is expressed at higher levels in Ca^{2+}-induced differentiated keratinocytes. Sequence alignment of N-terminus of cornulin and S100A8 protein showed that residues involved in the Ca^{2+} binding (Ishikawa *et al*, 2000) are well conserved (Fig 2). Effectively, Ca^{2+}-binding assay proved that EF-hand domains of cornulin are functional as observed for the other "fused" proteins, trichohyalin, repetin, and profilaggrin, which also bind calcium (Lee *et al*, 1993; Markova *et al*, 1993; Presland *et al*, 1995).

Previous studies have shown that expression of late markers of differentiation could be mediated/regulated by RA (Asselineau *et al*, 1989; Hohl *et al*, 1991, 1995; Krieg *et al*, 1997). The position of a RAR site in the promoter region of *c1orf10* suggests that cornulin expression is likely mediated by RA. Moreover, the presence of an AP1 site indicates that cornulin expression could be regulated through a PKC-mediated pathway. In fact, PKC has been shown to be a fundamental regulator of the coordinated changes in keratinocyte gene expression that occur during the spinous to granular cell transition in the epidermis (Dlugosz and Yuspa, 1993). Interestingly, several AP1 sites were described to be functional and essential for high-level transcriptional activity of many late markers (DiSepio *et al*, 1995; Welter *et al*, 1995). The identical AP1 site in both cornulin and profilaggrin promoters (Jang *et al*, 1996) suggests a similar mechanism for their transcriptional regulation.

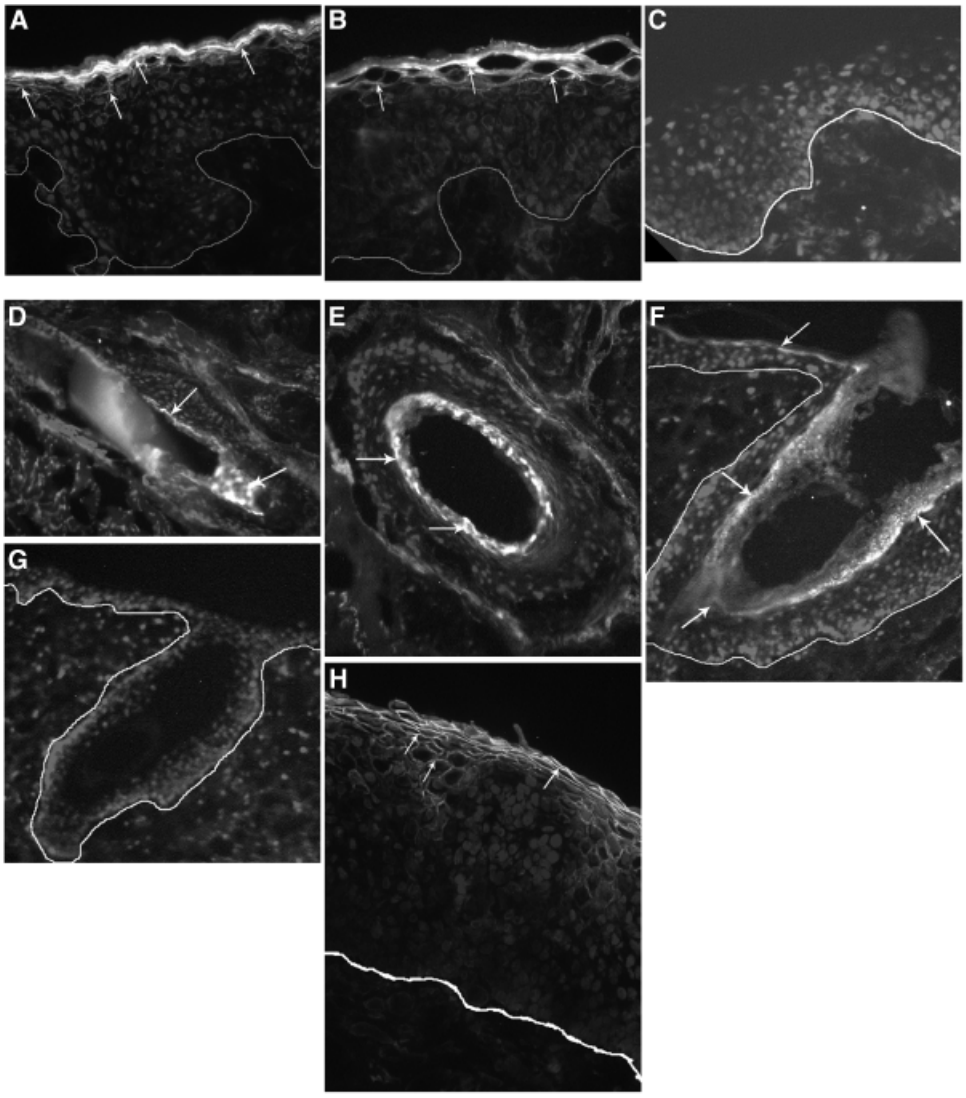

Figure 7
Human cornulin is expressed in the upper layers of epidermis. (*A*) Immunodetection was performed on frozen foreskin (*A–C*), scalp (*D–G*) and esophagus (*H*) with anti-cornulin antibody SZ1229 in adjacent perpendicular sections. The nucleus was stained with Hoechst 33258 dye. The basal layer is indicated (*solid line*). In the foreskin, the staining appears in the granular and lower cornified cell layers with a peripheral localization (*arrows*, *A*, *B*). Cornulin is expressed in the outer most layer of scalp skin and in the inner root sheat of the hair follicle (*arrows*, *D–F*). In normal esophagus, the staining is localized in the upper spinous and granular layers with strong peripheral localization (*arrows*, *H*). Control sections with secondary antibody alone show no staining (*C* and *G*).

Results obtained by western blotting with affinity-purified polyclonal anti-cornulin antibody indicate that cornulin might undergo post-translational modifications because the apparent molecular weight is about 70 kDa, whereas the calculated weight is about 53 kDa. This is consistent with *in silico* prediction results (not shown), indicating that five Thr and 14 Ser residues could be phophorylated and two Asn residues could be N-glycosylated.

The final conclusion of whether cornulin is a substrate of TG needs further investigations. Co-transfection assays in HeLa cells, using both cornulin and TG 1, showed no co-localization at the cell periphery suggesting that cornulin is not a substrate of this enzyme. However, cornulin is mainly localized in the periphery of cells *in vivo*. To explain these results, we hypothesize that HeLa cells do not contain "cross-linking" partners to whom cornulin could bind. Residues important in the cross-linking reaction are lysine and glutamine, but cornulin is poor in lysine residues (2.6%) compared with SPRR4 (15.6%). Thus, cross-linkage of cornulin to itself may be limited.

In conclusion, because of its genomic localization and organization, its structural features, Ca^{2+}-mediated regulation, and its expression in upper layers of the epidermis, we provide evidence that cornulin is a new member of the "fused gene" family and a new marker of late epidermal differentiation.

Materials and Methods

Human keratinocytes culture Foreskin keratinocytes were used for RNA and protein extractions. They were cultured in defined keratinocyte-SFM medium (Gibco Basel, Switzerland) supplemented with growth complement (Gibco) in a 37°C humidified incubator with 5% CO_2 atmosphere. When cells reached 90% confluence, they were either harvested (d0) or differentiated by a Ca^{2+}-shift from 0.1 to 1.2 mM, during 3 and 7 d (d3 and d7, respectively).

RNA extraction Total RNA was isolated from cultured cells and human foreskin, using the guanidine thiocyanate method (Chomczynski and Sacchi, 1987). RNA concentration was measured by OD 260 nm.

Semi-quantitative analysis of cornulin transcripts by RT-PCR RT-PCR experiments were carried out with total RNA from different human tissues using the One-Step RT-PCR kit (Qiagen Hambrechtikon, Switzerland) according to the manufacturer's instructions. Total RNA were first DNAse-treated as follows: 1 μg total RNA was incubated with 1 U DNAse I (Gibco), in a 10 μL final volume for 15 min at room temperature. The enzyme was then inactivated by addition of 1 μL 25 mM EDTA and incubation at 65°C for 10 min. First-strand cDNA were synthesized in a 50 μL reaction

containing 10 μL of the DNAse-treated RNA mix, 1 × PCR buffer, 0.4 mM dNTP, 0.6 mM primers of cornulin or actin, 2 μL enzyme mix and RNAse free water. RT was performed at 50°C for 30 min. PCR was carried out at an annealing temperature of 52°C. The RT-PCR products were resolved by 1% agarose gel electrophoresis. The lengths of the PCR products of cornulin and actin were 323 and 650 bp, respectively. The primer sequences of cornulin were as follows: upstream primer, 5′-CCCGACTCCAATGTCCAGTA; downstream primer, 5′-GCATTAGGGTAGATGGGGCA; for actin: 5′-TTCCTTCCTGGGCAT GGA GTC and 5′-GAGAAGTGGGGTGG CTTTTAG.

Isolation of human cornulin cDNA According to the sequence deposited in Genbank (AF077831), full-length human cornulin cDNA was obtained by RT-PCR from foreskin RNA. The upstream and downstream primers were: 5′-CCAGATCT ATGCCTCAGTTAC TGCAAAAC and 5′-TCGGTACCTCATGGCTTGGTGCTTCTCAA. This PCR product was cloned in pEGFP-C1 vector (Clontech, Basel, Switzerland), digested by *Asp*718 and *Bgl*II. Construct was verified by sequencing (Synergene, Schlieren, Switzerland).

Constructs and transfection procedures The different GFP-contructs used in this study were: (1) full-length cornulin, as described above; (2) cornulin ΔEF-hand (cornulin res 90–495), obtained by RT-PCR using pEGFP-C1-C1orf10 as DNA template and the primers 5′-ATGCCTCAGTTACTGCAAAAC (upstream) and 5′-ATGTCGACTCATGGCTTGGTGCTTCTCAAG (downstream) and cloned in EcoRI/SalI digested pEGFP-C2 vector; 3) similarly, EF-hand alone (cornulin res 1–90), cloned in the BglII/Asp718 digested pEGFP-C1, was obtained by PCR using pEGFP-C1-C1orf10 as DNA template and the primers 5′-CCAGATCTATGCCTCGTTCTGC AAAAC (upstream) and 5′-ACGGTACCAACTTTAAACACTAAGAC-CAGGA (downstream). PCR reactions were carried out at an annealing temperature of 57°C. HeLa cells were transfected with these DNA constructs at 70%–80% of confluence using Lipofectamine 2000 and Reagent Plus (Invitrogen, Basel, Switzerland), according to the manufacturer's instructions. Additionally, full-length cornulin was cloned into the pCruz-HA-b (Santa Cruz, Heidelberg, Germany) and pBudCE4 (Invitrogen) vectors to generate N-terminus-HA and C-terminus-V5 tagged cornulin, respectively. All the constructs were sequenced (Synergene).

Protein extraction Cells were first washed with phosphate-buffered saline (PBS), then harvested using buffer A (10 mM Tris HCl pH 7.4, 5 mM EDTA, 1 × complete protease inhibitor cocktail (Roche, Basel, Switzerland) and 1 mM PMSF) and transferred into an Eppendorf tube. Cells were sonicated on ice, three times for 10 s and centrifuged at 14,326 g for 30 min. The supernatant (cytosolic fraction) was transferred into a new tube. Protein concentrations were determined by a Bradford assay (BioRad, Reinach, Switzerland) using bovine serum albumin (BSA) as standard (Bradford, 1976).

Bacterial expression Coding sequence of cornulin and cornulin ΔEF-hand was subcloned into pET28a(+) (Novagen, Dietikon, Switzerland) yielding plasmid p28C and p28CΔ, respectively. Recombinant proteins were expressed in Rosetta 2(DE3)pLysS bacteria (Novagen) by induction with 1 mM IPTG at an OD_{600} of 0.6–0.8. Cultures were continued for an additional 3 h at 37°C. Induced bacteria were lysed (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1 mg per mL lysozyme) on ice for 30 min and submitted to three freeze–thaw cycles. Lysate was then sonicated 2 × 10 s on ice and centrifuged at 13,000 r.p.m. for 10 min at 4°C. The supernatant and pellet were defined as the soluble and particulate fraction, respectively.

Antibodies A polyclonal rabbit antibody (SZ1229) was elicited against a synthetic peptide of human cornulin (EATNDQ NRGTETHGQG) and affinity purified (Eurogentech, Seraing, Belgium). It was diluted at 1:2000 for immunoblotting and 1:100 for immunofluorescence.

Western blotting Human foreskin epidermis of young children was homogenized (Turrax). Protein extractions were performed as described above. Proteins (10 μg) were heated at 95°C for 5 min in SDS-sample buffer and loaded onto 10% polyacrylamide gels, electrophoresed, and transferred onto Hybond ECL transfer membranes (Amersham Pharmacia Biotech, Staufan, Germany). After incubating for 2 h in blocking buffer (20 mM Tris HCl, 150 mM NaCl, 0.5% Tween 20, 2% BSA and 2% non-fat dry milk powder), the membranes were probed with primary antibodies diluted in blocking buffer and detected with a horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham, Freiburg, Germany) using the ECL western-blotting detection reagents (Amersham Pharmacia Biotech).

Northern blot and *in situ* hybridization cDNA probe (~ 320 bp) was obtained by RT-PCR from foreskin total RNA using 3′ UTR-specific primers described above and cloned into the vector pGEM-T easy (Promega, Wallisellen, Switzerland). For northern blot analysis, the probe was labeled using Random Primed DNA Labeling Kit (Roche) according to the manufacturer's instructions. Total RNA from both proliferating and differentiating cultured keratinocytes was fractionated (12 μg per lane) by electrophoresis on 6% formaldehyde, 1.3% agarose gel in 1 × MOPS buffer. Separated RNA was then trans-blotted to a nylon membrane (Zeta-probe GT genomic, BioRad) and cross-linked by UV irradiation. The membrane was pre-hybridized (0.25 M Na_2HPO_4, 7% SDS, 1 mM EDTA, 10 mg per mL salmon sperm) for 1 h at 65°C. It was then hybridized with northern blot probe, in an agitating incubator for 18 h at 65°C. Finally, the membrane was washed for 10 min at 42°C with 0.2 × SSC/1% SDS, and for 30 min at 65°C with 0.2 × SSC/0.1% SDS. After washing, blot was exposed to X-OMAT AR film (Kodak, Renens, Switzerland) for 1 wk at −70°C.

For *in situ* hybridization, digoxigenin (DIG)-labeled riboprobes were synthesized with DIG RNA labeling kit (SP6/T7) according to the manufacturer's protocol (Roche). Plasmid was linearized with *Nco*I or *Spe*I and DIG-cRNA synthesized with SP6 or T7 polymerase for sense probe or anti-sense probe, respectively. Foreskin tissue samples were fixed in 4% paraformaldehyde dissolved in PBS for 24 h and embedded in paraffin. Sections (5 μm) were rehydrated, and incubated with 400 ng per mL of equivalently DIG-labeled sense or anti-sense riboprobe diluted in 5 × SSC, 2 × Denhardt's solution, 2.5 × dextran sulfate, 50% formamide, and 0.2 mg per mL heat-denatured salmon sperm DNA at 58°C overnight. Sections were washed with 2 × SSC, 50% formamide at 60°C for 20 min (twice), 1 × SSC at 60°C for 20 min (twice), and 0.2 × SSC at 60°C for 30 min (once). Hybridized DIG-riboprobes were detected with sheep anti-DIG Fab fragments conjugated to alkaline phosphatase, diluted 1:2500 in 0.5 × blocking solution (Roche) supplemented with 1% normal sheep serum. Finally, sections were incubated for 4 d with BMpurple (Roche) diluted in developing buffer containing 1 mM levamisole (for more details, see Roche instruction manual and de Viragh *et al*, 1994).

Immunofluorescence microscopy Immunofluorescence was performed on frozen tissue samples embedded in Tissue Tek (Sakura, Horgen, Switzerland) and cut into 5 μm thick sections, or on transfected cells, grown on glass coverlips, fixed with ice-cold methanol. Samples were washed 2 × 10 min in PBS and 2 min in water. They were blocked in PBS containing 12% BSA for an hour and then incubated with primary antibody overnight at 4°C in PBS-12% BSA. The primary antibody was detected by fluorescein isothiocyanate-labeled pig anti-rabbit IgG incubated for 30 min at room temperature. Multiple washes in PBS were carried out between each step. For DNA staining, samples were incubated in PBS, 0.1% NP-40, and 0.01% Hoechst 33258 (Sigma, Buchs, Switzerland) for 2 min. Samples were finally mounted using DAKO fluorescent mounting medium. Fluorescence was evaluated using an epifluorescence Zeiss Axisocope microscope (Carl Zeiss, Obeskocher, Germany) and digital images were collected with an RTcolor SPOT CCD camera (Diagnostic Instruments, Houston,

Texas). The protocol was the same for the transfected cells except that the primary antibody was detected using biotinylated horse anti-mouse IgG (Vector Laboratories, Burlingame, California) followed by streptavidine conjugated to Texas Red (Amersham). Sections without primary antibody were used as negative controls. Pictures treatment was made with Photoshop 6 software (Adobe).

Bioinformatics analysis Homology of human cornulin sequence to other species proteins was determined with Genbank and Ensembl BLAST databases (http://www.ncbi.nlm.nih.gov/BLAST/ and http://www.ensembl.org).

Study of the 5′ upstream DNA sequence and intron I was made by using AliBaba 2 program (http://www.gene-regulation.com). Investigation of the secondary structure and modification of the protein was carried out with tools available at ExPASy molecular biology server (http://www.expasy.org). Multiple alignments were made with the AlignX program of the Vector NTI suite (Informax, Basel, Switzerland).

Statement Biopsies were obtained after written informed consent and the study, approved by the medical ethical committee of the University of Lausanne Medical Faculty, was conducted according to the Declaration of Helsinki Principles.

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References

Asselineau D, Bernard BA, Bailly C, Darmon M: Retinoic acid improves epidermal morphogenesis. Dev Biol 133:322–335, 1989

Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 72:248–254, 1976

Cabral A, Sayin A, de Winter S, Fischer DF, Pavel S, Backendorf C: SPRR4, a novel cornified envelope precursor: UV-dependent epidermal expression and selective incorporation into fragile envelopes. J Cell Sci 114: 3837–3843, 2001

Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal Biochem 162:156–159, 1987

de Viragh PA, Huber M, Hohl D: Involucrin mRNA is more abundant in human hair follicles than in normal epidermis. J Invest Dermatol 103:815–819, 1994

DiSepio D, Jones A, Longley MA, Bundman D, Rothnagel JA, Roop DR: The proximal promoter of the mouse loricrin gene contains a functional AP-1 element and directs keratinocyte-specific but not differentiation-specific expression. J Biol Chem 270:10792–10799, 1995

Dlugosz AA, Yuspa SH: Coordinate changes in gene expression which mark the spinous to granular cell transition in epidermis are regulated by protein kinase C. J Cell Biol 120:217–225, 1993

Fuchs E, Green H: Changes in keratin gene expression during terminal differentiation of the keratinocyte. Cell 19:1033–1042, 1980

Haftek M, Serre G, Mils V, Thivolet J: Immunocytochemical evidence for a possible role of cross-linked keratinocyte envelopes in stratum corneum cohesion. J Histochem Cytochem 39:1531–1538, 1991

Hohl D, de Viragh PA, Amiguet-Barras F, Gibbs S, Backendorf C, Huber M: The small proline-rich proteins constitute a multigene family of differentially regulated cornified cell envelope precursor proteins. J Invest Dermatol 104:902–909, 1995

Hohl D, Lichti U, Breitkreutz D, Steinert PM, Roop DR: Transcription of the human loricrin gene *in vitro* is induced by calcium and cell density and suppressed by retinoic acid. J Invest Dermatol 96:414–418, 1991

Ishikawa K, Nakagawa A, Tanaka I, Suzuki M, Nishihira J: The structure of human MRP8, a member of the S100 calcium-binding protein family, by MAD phasing at 1.9 A resolution. Acta Crystallogr D 56 (Part 5):559–566, 2000

Jang SI, Steinert PM, Markova NG: Activator protein 1 activity is involved in the regulation of the cell type-specific expression from the proximal promoter of the human profilaggrin gene. J Biol Chem 271:24105–24114, 1996

Kalinin AE, Kajava AV, Steinert PM: Epithelial barrier function: Assembly and structural features of the cornified cell envelope. Bioessays 24:789–800, 2002

Krieg P, Schuppler M, Koesters R, Mincheva A, Lichter P, Marks F: Repetin (Rptn), a new member of the "fused gene" subgroup within the S100 gene family encoding a murine epidermal differentiation protein. Genomics 43: 339–348, 1997

Lee SC, Kim IG, Marekov LN, O'Keefe EJ, Parry DA, Steinert PM: The structure of human trichohyalin. Potential multiple roles as a functional EF-hand-like calcium-binding protein, a cornified cell envelope precursor, and an intermediate filament-associated (cross-linking) protein. J Biol Chem 268:12164–12176, 1993

Lioumi M, Olavesen MG, Nizetic D, Ragoussis J: High-resolution YAC fragmentation map of 1q21. Genomics 49:200–208, 1998

Luo A, Kong J, Hu G, *et al*: Discovery of Ca(2+)-relevant and differentiation-associated genes downregulated in esophageal squamous cell carcinoma using cDNA microarray. Oncogene 23:1291–1299, 2004

Makino T, Takaishi M, Morohashi M, Huh NH: Hornerin, a novel profilaggrin-like protein and differentiation-specific marker isolated from mouse skin. J Biol Chem 276:47445–452, 2001

Marenholz I, Zirra M, Fischer DF, Backendorf C, Ziegler A, Mischke D: Identification of human epidermal differentiation complex (EDC)-encoded genes by subtractive hybridization of entire YACs to a gridded keratinocyte cDNA library. Genome Res 11:341–355, 2001

Markova NG, Marekov LN, Chipev CC, Gan SQ, Idler WW, Steinert PM: Profilaggrin is a major epidermal calcium-binding protein. Mol Cell Biol 13:613–625, 1993

Maruyama K, Mikawa T, Ebashi S: Detection of calcium binding proteins by 45Ca autoradiography on nitrocellulose membrane after sodium dodecyl sulfate gel electrophoresis. J Biochem (Tokyo) 95:511–519, 1984

Marvin KW, George MD, Fujimoto W, Saunders NA, Bernacki SH, Jetten AM: Cornifin, a cross-linked envelope precursor in keratinocytes that is down-regulated by retinoids. Proc Natl Acad Sci USA 89:11026–11030, 1992

Mehrel T, Hohl D, Rothnagel JA, *et al*: Identification of a major keratinocyte cell envelope protein, loricrin. Cell 61:1103–11012, 1990

Mischke D, Korge BP, Marenholz I, Volz A, Ziegler A: Genes encoding structural proteins of epidermal cornification and S100 calcium-binding proteins form a gene complex ("epidermal differentiation complex") on human chromosome 1q21. J Invest Dermatol 106:989–992, 1996

Moll R, Franke WW, Schiller DL, Geiger B, Krepler R: The catalog of human cytokeratins: Patterns of expression in normal epithelia, tumors and cultured cells. Cell 31:11–24, 1982

Presland RB, Bassuk JA, Kimball JR, Dale BA: Characterization of two distinct calcium-binding sites in the amino-terminus of human profilaggrin. J Invest Dermatol 104:218–223, 1995

Presland RB, Haydock PV, Fleckman P, Nirunsuksiri W, Dale BA: Characterization of the human epidermal profilaggrin gene. Genomic organization and identification of an S-100-like calcium binding domain at the amino terminus. J Biol Chem 267:23772–23781, 1992

South AP, Cabral A, Ives JH, *et al*: Human epidermal differentiation complex in a single 2.5 Mbp long continuum of overlapping DNA cloned in bacteria integrating physical and transcript maps. J Invest Dermatol 112:910–918, 1999

Steinert PM, Marekov LN: The proteins elafin, filaggrin, keratin intermediate filaments, loricrin, and small proline-rich proteins 1 and 2 are isodipeptide cross-linked components of the human epidermal cornified cell envelope. J Biol Chem 270:17702–17711, 1995

Welter JF, Crish JF, Agarwal C, Eckert RL: Fos-related antigen (Fra-1), junB, and junD activate human involucrin promoter transcription by binding to proximal and distal AP1 sites to mediate phorbol ester effects on promoter activity. J Biol Chem 270:12614–12622, 1995

Xu Z, Wang MR, Xu X, *et al*: Novel human esophagus-specific gene c1orf10: cDNA cloning, gene structure, and frequent loss of expression in esophageal cancer. Genomics 69:322–330, 2000